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MicroRNA-196a-5p targeting LRP1B modulates phenotype of thyroid carcinoma cells

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Abstract

Introduction: Thyroid cancer (TC) is a common endocrine malignancy, comprising nearly one-third of all head and neck malignancies worldwide. MicroRNAs (miRNAs) have been implicated in the malignant progression of multiple cancers; however, their contribution to thyroid diseases has not been fully explored.

Material and methods: This study aimed to illustrate the regulatory mechanism of microRNA-196a-5p in TC progression and to investigate whether microRNA-196a-5p affects progression of TC cells by targeting low-density lipoprotein receptor-associated protein 1B (LRP1B). MicroRNA-196a-5p and LRP1B expression status in TC cells and normal human thyroid cells was detected by quantative reverse transcription polymerase chain reaction (qRT-PCR) and western blot. Dual-luciferase reporter assay, cell counting kit-8 (CCK-8) assay, scratch healing assay, and Transwell assay were also performed.

Results: The results showed that microRNA-196a-5p expression was up-regulated and LRP1B expression was down-regulated in TC cells. In addition, the upregulation of microRNA-196a-5p facilitated progression of TC cells. Silencing microRNA-196a-5p led to the opposite results. Dual-luciferase reporter assay offered evidence for microRNA-196a-5p targeting LRP1B in TC. MicroRNA-196a-5p could target LRP1B to facilitate proliferation, invasion, and migration of TC cells.

Conclusion: Overall, this study revealed that microRNA-196a-5p may be a cancer-promoting microRNA that plays an important role in TC progression.

Key words: *microRNA-196a-5p; LRP1B; thyroid carcinoma; proliferation; migration; invasion*

Introduction

Thyroid cancer (TC) is one of the most common cancers in the world, and its incidence is increasing year by year [1, 2]. Multiple kinase inhibitor drugs are standard treatment for TC, but they are accompanied by some adverse reactions. Targeted therapies that drive molecular changes improve the overall safety of standard therapy [3]. Despite great progress in the treatment of TC, the survival rate of patients with advanced TC is still low [4]. Further understanding of the pathogenesis of this disease is required to develop more effective treatment options.

MicroRNAs are endogenous 19–24 nt-length non-coding RNA that can directly bind to mRNA and affect gene expression at the post-transcriptional level [5, 6]. More and more studies have confirmed that microRNAs regulate gene expression through direct interaction with complementary sequences on target mRNAs [5, 7]. MicroRNAs participate in a variety of biological behaviours, such as tumourigenesis and cancer metastasis [8–10]. For example, microRNA-31 is involved in the regulation of circRNA_0000140 on the growth and metastasis of oral squamous cell carcinoma [11]. MicroRNA-146b-5p hampers metastasis and tumourigenesis of gallbladder cancer by targeting Toll-like receptor 4 via the nuclear factor-κB pathway [12]. MicroRNA-133b can suppress colorectal cancer metastasis by lncRNA LUCAT1 [13]. MicroRNA-196a-5p is a conserved microRNA derived from its precursor microRNA-196a [14]. MicroRNA-196a-5p has been studied in diseases including non-small cell lung cancer [15], cataracts [16], and gastric cancer [17]. However, in TC, the effect and specific mechanism of microRNA-196a-5p remain unclear.

Low-density lipoprotein receptor-associated protein 1 (LRP1) is a multifunctional receptor involved in many biological processes, including signal transduction, lipoprotein metabolism, regulation of vascular permeability and tension, cell growth, migration, and apoptosis [18]. LRP1B is a tumour suppressor that encodes LDL receptor [19]. LRP1B was originally discovered based on a homozygous deletion analysis in human lung cancer cell lines [20]. LRP1B is a member

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of the low-density lipoprotein receptor (LDLR) family. LRP1 and LRP1B share 86% of mRNA and 52% of amino acid fragments, which enables the 2 receptors to have some similar functions [18, 20]. A study by Li et al. [21] showed that high expression of LRP1B can mediate lipid metabolism of hepatocellular carcinoma cells. Zhen et al. [22] discovered that LRP1B plays an imperative role in promoting hepatocellular carcinoma progression by regulating the PERK-ATF4-CHOP signalling pathway. However, there are few studies on LRP1B in TC, and further research should be carried out.

We found that microRNA-196a-5p in TC cells was increased relative to that in normal thyroid cells, and microRNA-196a-5p could significantly promote progression of TC cells. Also, dual-luciferase reporter gene assay and quantative reverse transcription polymerase chain reaction (qRT-PCR) assay confirmed that LRP1B was the direct target of microRNA-196a-5p in TC.

Material and methods

Bioinformatics approaches

TCGA-THCA mRNA (normal: 58, tumour: 510) and microRNA expression data (normal: 59, tumour: 514) were acquired from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). EdgeR was used for differential analysis ($|\log FC| > 2$, padj < 0.01) to obtain 62 differentially expressed microRNAs and 1088 differentially expressed mRNAs. The high- and low-expression groups were divided based on median value. Survival analysis of microRNA was then performed. The TargetScan (http://www.targetscan.org/vert_72/) database and miRDB (http://mirdb.org/) database were used for target gene prediction of the target microRNA, then the predicted results were overlapped with differential mRNAs to obtain the differential mRNA with targeted binding sites of the target microRNA. The correlation between microRNA and mRNA was analysed through Pearson correlation analysis.

Cell culture and transfection

The cell lines used in this study provided by BeNa Culture Collection were as follows: human normal thyroid follicular epithelial cell line Nthy-ori3-1 (BNCC340487) and human TC cell lines K-1 (BNCC337627), TPC-1 (BNCC337912), SW579 (BNCC100702), and FTC133 (BNCC352059). Dulbecco's modified Eagle medium (DMEM) plus 10% foetal bovine serum (FBS) was used for cell incubation. The temperature was 37°C, and the culture container was a humidified incubator of 5% CO_2.

The sequences and plasmids used in this study for transfection were synthesized by Guangzhou RiboBio Co., LTD. They were microRNA-196a-5p mimic (microRNA-mimic), mimic NC, microRNA-196a-5p inhibitor (microRNA-inhibitor), inhibitor NC, pcDNA3.1-LRP1B plasmid (oe-LRP1B), and empty pcDNA3.1 plasmid (oe-NC). Lipofectamine 2000 (Invitrogen, USA) was introduced for cell transfection.

Quantative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was separated from cells using TRIzol reagent (Invitrogen, United States). Then, a PrimeScript TM reverse transcriptase kit (TaKaRa, Japan) was utilized to make the RNA transcribed into cDNA. U6 and GAPDH were used as the standards for quantifying relative expression, respectively, and the $2^{-\Delta\Delta Ct}$ method was used for calculation. The primers are shown in Table 1.

Cell counting kit-8 (CCK-8) assay

TC cells TPC-1 and SW579 were seeded into 96-well plates (2×10^3 cells/well). After 0, 24, 48, 72, and 96 h, the cells were added with 10 μL CCK-8 solution (CK04; Dojindo Laboratories, Japan) and maintained at 37°C and 5% CO_2 for another 2 h. Absorbance was measured at 450 nm at a specified time point, with the absorbance value measured on the first day as a control.

Scratch healing assay

When cell fusion efficiency reached about 90%, the cell monolayer was scratched using a 200 μL pipette in each well. The cells were rinsed 3 times with phosphate buffer saline (PBS) to remove the exfoliated cells. The cells were then incubated in serum-free medium under the same conditions for 24 h. At each time point (0, 24 h), the scratch area was photographed.

Transwell invasion assay

Transwell chambers with pore diameter of 8 μm (Corning, United States) were introduced to examine cell invasion. 1×10^5 cells were re-suspended in 200 μL FBS-free medium and inoculated in the upper chamber precoated with Matrigel. The lower chamber was supplemented with complete medium containing 10% FBS. After incubation for 48 h at 37°C and 5% CO_2, the successfully invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, the cell numbers were counted under 5 randomly selected fields.

Western blot

Total proteins were isolated from the cells using RIPA (Thermo Fisher, United States). Protein concentration was determined by the Bradford method. An equal amount of proteins was separated by SDS-PAGE at 220 V and transferred to a PVDF membrane. Membrane was blocked with 5% bovine serum albumin for 2 h. The membrane was probed with primary antibodies rabbit anti-LRP1B (Thermo Fisher, USA) and rabbit anti-GAPDH (Abcam, UK) overnight, and then with secondary antibody horseradish peroxidase (HRP)-coupled IgG H&L (Abcam, United Kingdom) for 1–2 h. Electrochemiluminescence (ECL) was used to observe the prints.

Table 1. *Quantative reverse transcription polymerase chain reaction (qRT-PCR) primer sequences*

Gene	Primer sequences (5′ → 3′)	
microRNA-196a-5p	UAGGUAGUUUCAUGUUGUUGGG	
U6	F: CTCGCTTCGGCAGCACA	R: AACGCTTCACGAATTTGCGT
LRP1B	F: CCCCAAAGAGCAGCAAGTCT	R: CCAAGAGGACGAGAGGCACA
GAPDH	F: TGCACCACCAACTGCTTAGC	R: GGCATGGACTGTGGTCATGAG

Dual-luciferase assay

The constructed reporter vector LRP1B-WT (wild type) or LRP1B-MUT (mutant) and microRNA-mimic or mimic NC were co-transfected into TPC-1 and SW579 cells through Lipofectamine 2000 (Invitrogen, United States). Luciferase activity was detected by a Cetro XS3 LB 960 microplate luminometer (Berthold, Germany) after 48 h of transfection.

Statistical analysis

Data analysis was performed using GraphPad Prism 6.0 (LaJolla, CA, United States). Each assay was repeated 3 times. Student's *t*-test was utilized for the 2-group comparison, and one-way analysis of variance was used for comparison between multiple groups. *P* value less than 0.05 represented a significant difference.

Results

MicroRNA-196a-5p is up-regulated in TC tissue and cells

MicroRNA data were acquired from TCGA database and analysed to obtain 62 microRNAs with differential expression. The red dots represent up-regulated genes, and the green dots represent down-regulated ones (Fig. 1A). Among them, microRNA-196a-5p has been reported to be linked with progression of colorectal cancer [23], breast cancer [24], glioma [25], and other cancers, except TC. Hence, microRNA-196a-5p was chosen as the target gene to carry out this investigation. In TCGA cohort, the expression level of microRNA-196a-5p in TC tissue was significantly higher than that of the control group (Fig. 1B), and the survival analysis results showed that poor prognosis of TC patients was associated with high microRNA-196a-5p expression (Fig. 1C). The level of microRNA-196a-5p in TC cell lines K-1, TPC-1, SW579, and FTC133 and normal cell line Nthy-ori3-1 was analysed by qRT-PCR, and the result suggested that microRNA-196a-5p in TC cells was significantly elevated (Fig. 1D). Tpc-1 and SW579 showed relatively high and low expres-

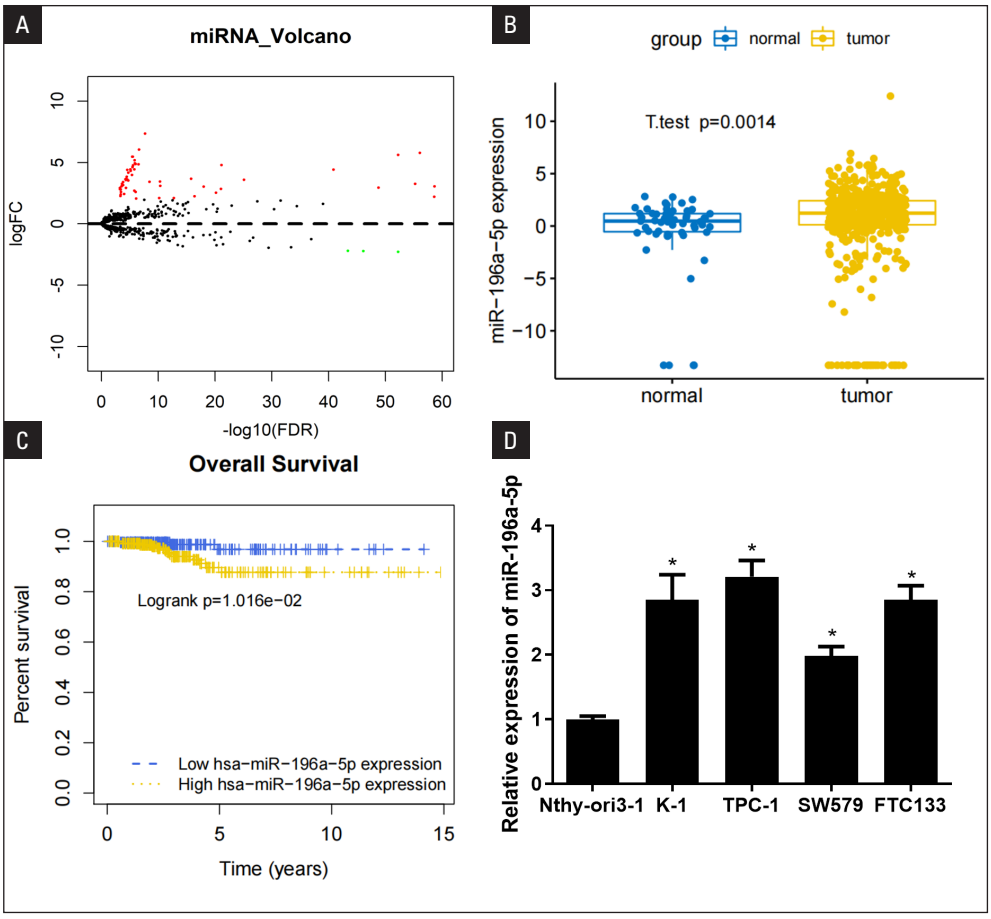

Figure 1. *Expression of microRNA-196a-5p is activated in thyroid cancer (TC) tissue and cells.* **A.** *Volcano plot of differential microRNAs in TCGA-THCA dataset; red indicates up-regulated microRNAs and green indicates down-regulated microRNAs;* **B.** *Comparison of microRNA-196a-5p expression level in normal and tumour tissues (yellow: tumour tissue; blue: normal tissue);* **C.** *Survival curves show the effect of microRNA-196a-5p expression on the prognosis of patients; the yellow line represents the high-expression group, and the blue line represents the low-expression group;* **D.** *MicroRNA-196a-5p level in normal cell line Nthy-ori3-1 and human TC cell lines K-1, TPC-1, SW579, and FTC133; *p < 0.05 vs. normal cells Nthy-ori3-1, the results were expressed as mean ± standard deviation (SD)*

sion of microRNA-196a-5p, respectively, so subsequent experiments were carried out in these 2 cell lines. In conclusion, microRNA-196a-5p was up-regulated in TC tissue and cells.

MicroRNA-196a-5p increases proliferation, migration, and invasion rates of TPC-1 and SW579 cells

Firstly, microRNA-196a-5p mimic and microRNA-196a-5p inhibitor were transfected into TPC-1 and SW579 cells. qRT-PCR result showed that the microRNA-196a-5p was prominently increased in TPC-1 and SW579 cells after transfection with microRNA-196a-5p mimic, and after transfection with microRNA-196a-5p inhibitor, microRNA-196a-5p in TPC-1 and SW579 cells was prominently reduced (Fig. 2A). Then a series of assays on cellular function were conducted. According to the CCK-8 assay, the survival ability of TPC-1 and SW579 cells was remarkably enhanced upon overexpression

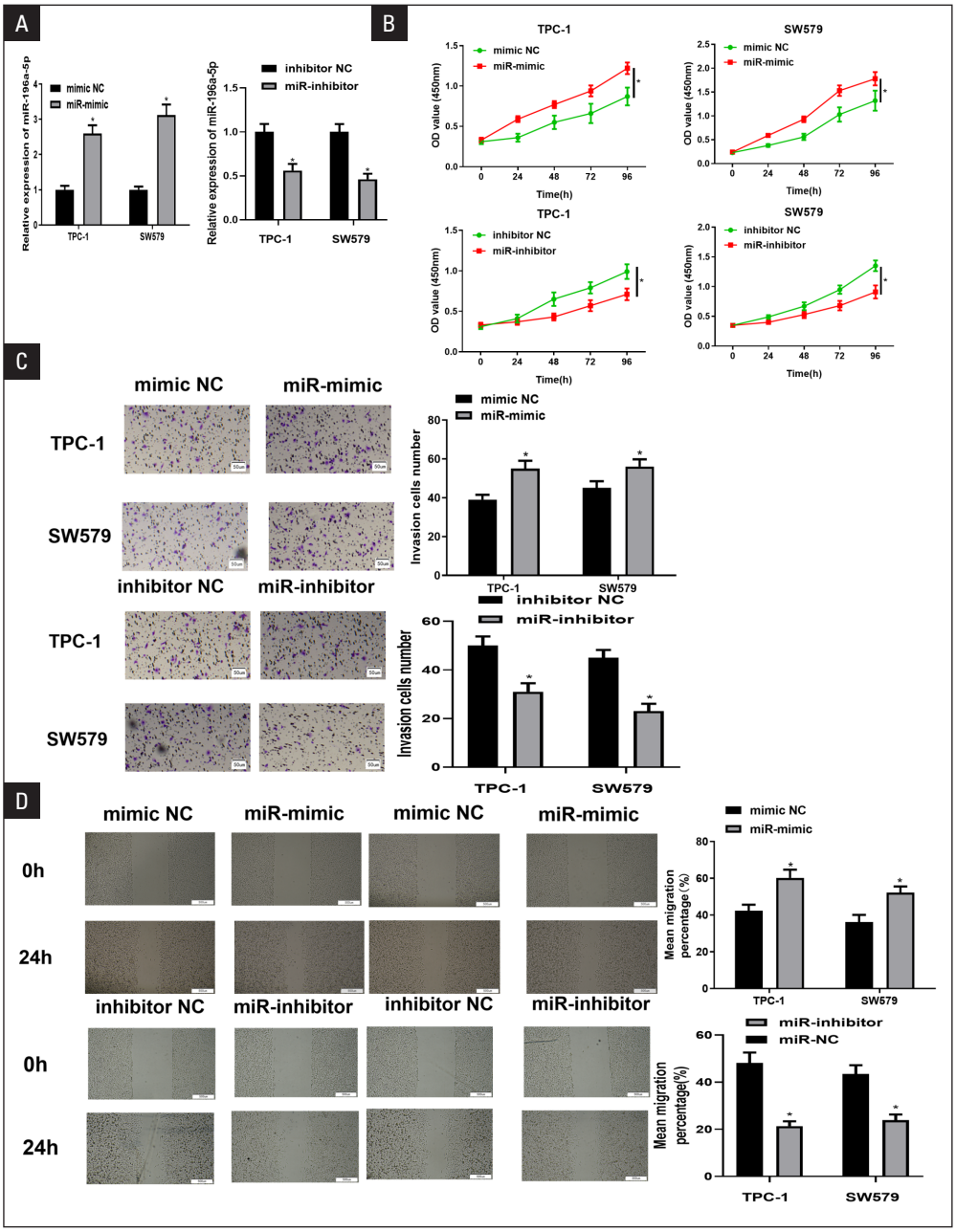

Figure 2. *MicroRNA-196a-5p increases the proliferation, migration, and invasion of TPC-1 and SW579 cells.* **A.** *Expression of microRNA-196a-5p in cells transfected with microRNA-mimic/inhibitor;* **B.** *Proliferation ability of cells transfected with microRNA-mimic/inhibitor;* **C.** *Invasive ability of cells transfected with microRNA-mimic/inhibitor (100×);* **D.** *Migratory ability of cells transfected with microRNA-mimic/inhibitor; *p < 0.05, the results were expressed as mean ± standard deviation (SD)*

of microRNA-196a-5p. Silencing microRNA-196a-5p evidently restrained proliferation of TPC-1 and SW579 cells (Fig. 2B). Additionally, the effect of overexpressed microRNA-196a-5p on the motility of TPC-1 and SW579 cells was also detected by Transwell invasion and scratch healing assays. It was revealed that invasion and migration of TPC-1 and SW579 cells were also significantly enhanced upon the overexpression of microRNA-196a-5p (Fig. 2C–D). Silencing microRNA-196a-5p repressed migration and invasion of TPC-1 and SW579 cells (Fig. 2C–D).

LRP1B is weakly expressed in TC cells, and is targeted by microRNA-196a-5p

Through bioinformatics analysis, 2 target genes with targeted binding sites of microRNA-196a-5p were obtained, namely LRP1B and NRXN1 (Fig. 3A), among which LRP1B has the highest correlation coefficient with microRNA-196a-5p (Fig. 3B), and the LRP1B level in TC tissue was decreased (Fig. 3C). Moreover, we analysed the clinicopathological information of TC patients and revealed that there were marked differences in LRP1B gene expression among different N stages, staging, and T stages (Fig. 3D–F). Subsequently, the expression of LRP1B in TC cell lines TPC-1, SW579, and normal cell line Nthy-ori3-1 was also detected. It was found that LRP1B mRNA expression was decreased in TC cell lines (Fig. 3G). Then, western blot was utilized with the result suggesting that the LRP1B protein level in TC cell lines was also decreased significantly (Fig. 3H). To further verify the binding interaction between microRNA-196a-5p and LRP1B, bioinformatics prediction was introduced firstly, which revealed that microRNA-196a-5p and LRP1B had binding sites (Fig. 3I). This prediction was further confirmed by dual-luciferase assay. MicroRNA-196a-5p mimic remarkably suppressed luciferase activity in the LRP1B-WT group while luciferase activity was not affected in the LRP1B-MUT group (Fig. 3J). In conclusion, LRP1B was underexpressed in TC cells, and a targeted relationship between LRP1B and microRNA-196a-5p was uncovered.

MicroRNA-196a-5p suppresses LRP1B expression

To explore the regulatory relationship between microRNA-196a-5p and LRP1B, LRP1B expression in TPC-1 and SW579 cells was also measured, and it was found that overexpression of microRNA-196a-5p inhibited expression of LRP1B at mRNA and protein levels while inhibiting expression of microRNA-196a-5p induced the opposite expression trend of LRP1B (Fig. 4AB). In conclusion, microRNA-196a-5p could inhibit the expression of LRP1B.

MicroRNA-196a-5p modulates TC progression by targeting LRP1B

To verify whether microRNA-196a-5p affects TC cell functions by suppressing LRP1B expression, a cell line with overexpression of LRP1B (mimic NC+oe-LRP1B) and a cell line with simultaneous overexpression of microRNA-196a-5p and LRP1B (microRNA-mimic+oe-LRP1B) were constructed to study the biological functions of cells. qRT-PCR and western blot showed that the LRP1B level in the mimic NC+oe-LRP1B group was increased notably, and it was restored when both microRNA-196a-5p and LRP1B were overexpressed (Fig. 5AB), which further indicated that microRNA-196a-5p negatively regulated LRP1B in TC cells. The CCK-8 assay result showed that LRP1B upregulation significantly suppressed proliferation of TC cells, while simultaneous overexpression of microRNA-196a-5p attenuated the inhibitory effect of overexpressed LRP1B on cell proliferation (Fig. 5C). Migration and invasion of cells in different treatment groups of 2 kinds of TC cells were further tested using scratch healing and Transwell assays, and it was found that after overexpressing LRP1B, both cell migratory and invasive abilities were significantly decreased, but when microRNA-196a-5p and LRP1B were overexpressed at the same time, the migratory and invasive abilities could recover a lot (Fig. 5DE). The results suggested that microRNA-196a-5p promoted the proliferation, migration, and invasion of TC cells by down-regulating LRP1B.

Discussion

Although TC is a mostly differentiated tumour with a low degree of malignancy, the incidence of TC is on the rise in recent years. Hence, exploring molecules that can influence the occurrence of TC is of great significance [26]. Considerable efforts have been made in genomics, epigenomics, and proteomics analysis to elucidate the mechanisms of TC occurrence and progression [27–29]. Studies have shown changes in non-coding RNAs (ncRNAs) at expression level, including microRNAs and long non-coding RNAs (lncRNAs), in papillary TC [30–32]. This study aimed mainly to investigate the biological function and molecular mechanism of microRNA-196a-5p in TC, with the hope of providing new targets for TC.

A study revealed that microRNA-196a-5p is abnormally expressed in multiple cancer tissues and is associated with the development of these cancers. For example, the study of Wei et al. found that microRNA-196a-5p is highly expressed in hepatocellular carcinoma and can promote proliferation, invasion, and migration of hepatocellular carcinoma Huh7 cells [33]. However, the role of microRNA-196a-5p in TC has

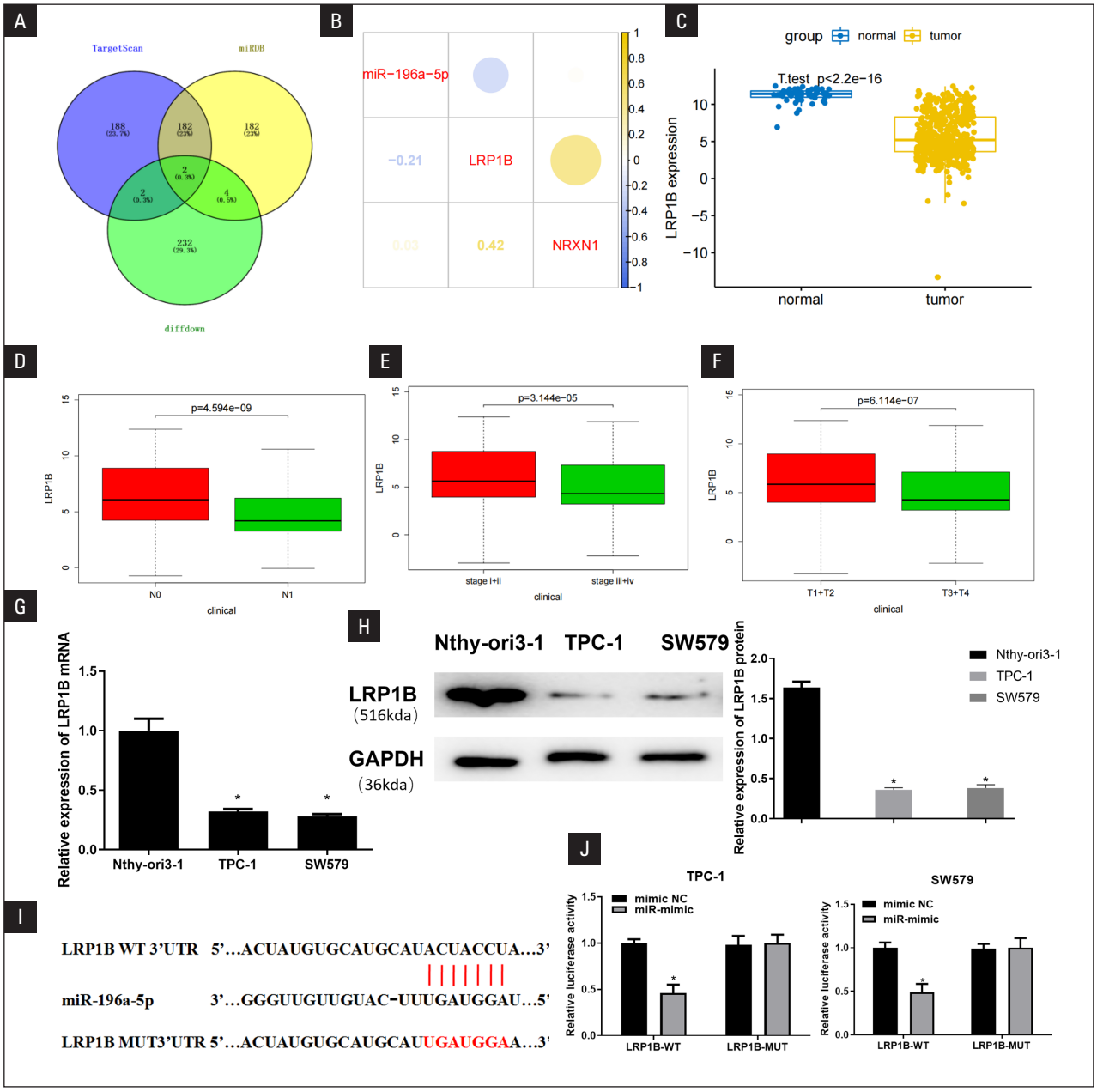

Figure 3. *Low-density lipoprotein receptor-associated protein 1B (LRP1B) expression is notably low in thyroid cancer (TC) cells.* **A.** *Venn diagram of predicted target genes of microRNA-196a-5p and differential mRNAs in TCGA;* **B.** *Correlation analysis between microRNA-196a-5p and 2 candidate target genes (LRP1B and NRXN1);* **C.** *The expression profile of LRP1B in normal group and tumour group;* **D–F.** *Correlation between LRP1B expression and clinicopathological features (including N stage, staging, and T stage);* **GH.** *qRT-PCR and western blot were used to detect the mRNA expression level and relative protein expression level of LRP1B in normal cell line Nthy-ori3-1 and TC cell lines TPC-1 and SW579;* **I.** *Predicted binding sequence of microRNA-196a-5p on LRP1B mRNA 3'-UTR; WT — wild type; MUT — mutant;* **J.** *Luciferase activity in different treatment groups of cancer cell lines TPC-1 and SW579; *p < 0.05 vs. normal cells Nthy-ori3-1; the results were expressed as mean ± standard deviation (SD)*

not been illustrated. Here, similar results were observed in TC. Through bioinformatics approaches and a series of cell experiments, we revealed that microRNA-196a-5p was up-regulated in TC cells and tissue, and facilitated progression of TC cells. These results suggest that microRNA-196a-5p played an important part in the malignant development of TC.

LRP1B interacts with various ligands and plays a wide range of roles in the normal function and development of cells. LRP1B can alter the expression of proteins associated with adhesion complex, and it is related to the transduction of cellular cargo transport signal [20]. In this study, we found that the level of LRP1B was down-regulated in TC cells, and its ex-

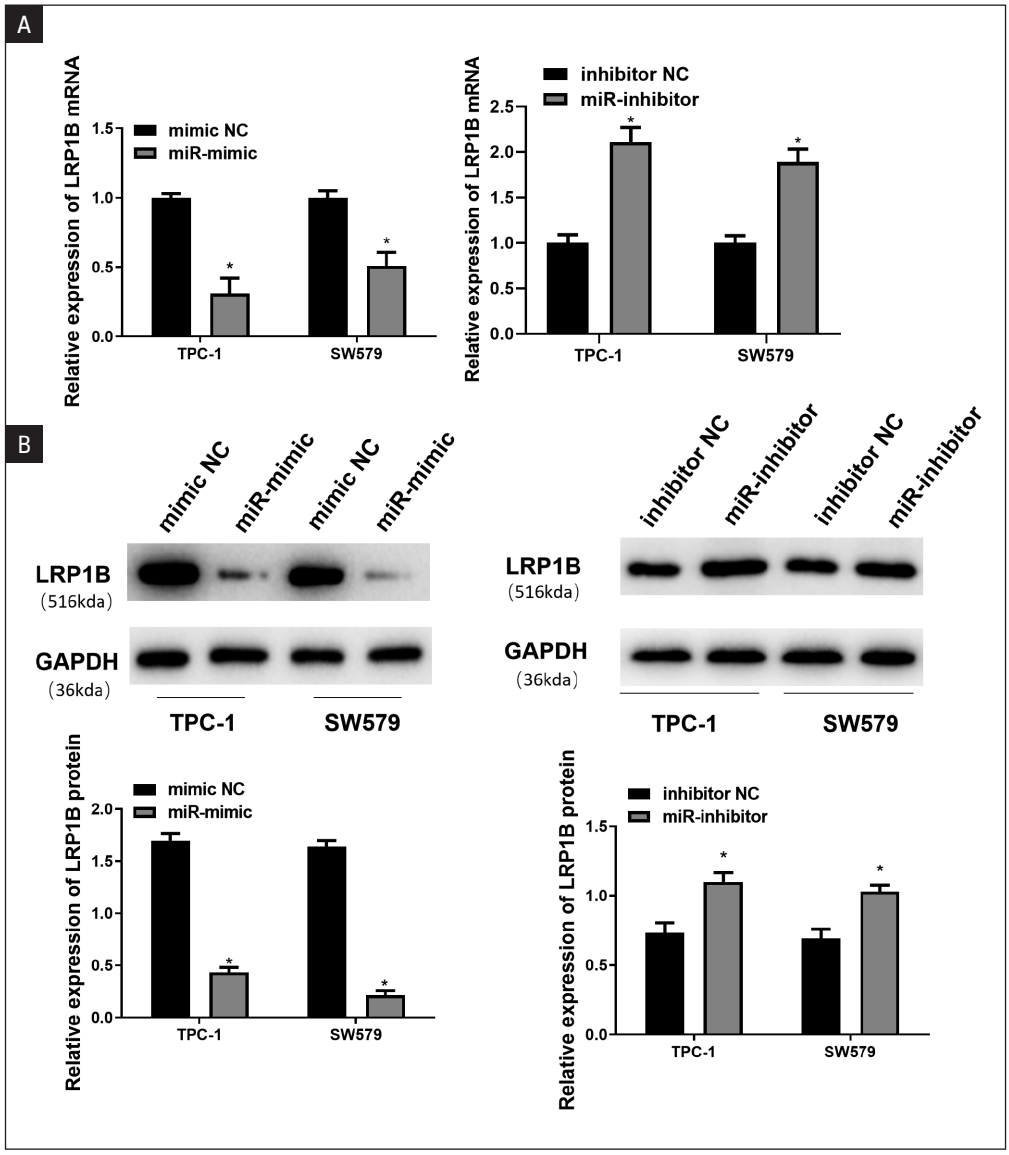

Figure 4. *MicroRNA-196a-5p targets low-density lipoprotein receptor-associated protein 1B (LRP1B).* **AB.** *Effects of microRNA-196a-5p on mRNA and protein expression of LRP1B; *p < 0.05 vs. mimic NC or inhibitor NC group, the results were expressed as mean ± standard deviation (SD)*

pression level was regulated by microRNA-196a-5p. Functional experiments revealed that the proliferation, migration, and invasion of TC cells were inhibited upon overexpression of LRP1B. In addition, we speculated that the low expression of LRP1B might be related to the poor prognosis of TC based on the inhibitory effect of overexpression of LRP1B on TC cell progression.

To sum up, this study investigated the functional role of microRNA-196a-5p in TC through in vitro assays, and the function of microRNA-196a-5p was realized by directly binding to the 3′-UTR of LRP1B. MicroRNA-196a-5p can be used as a new target for TC therapy. In order to further clarify the cancer-promoting effect of microRNA-196a-5p in TC and its potential mechanism, we will further confirm the carcinogenic effect of microRNA-196a-5p in vivo through animal experiments in the future and further explore the upstream and downstream molecular signalling pathways related to microRNA-196a-5p.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable

Availability of data and materials
The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

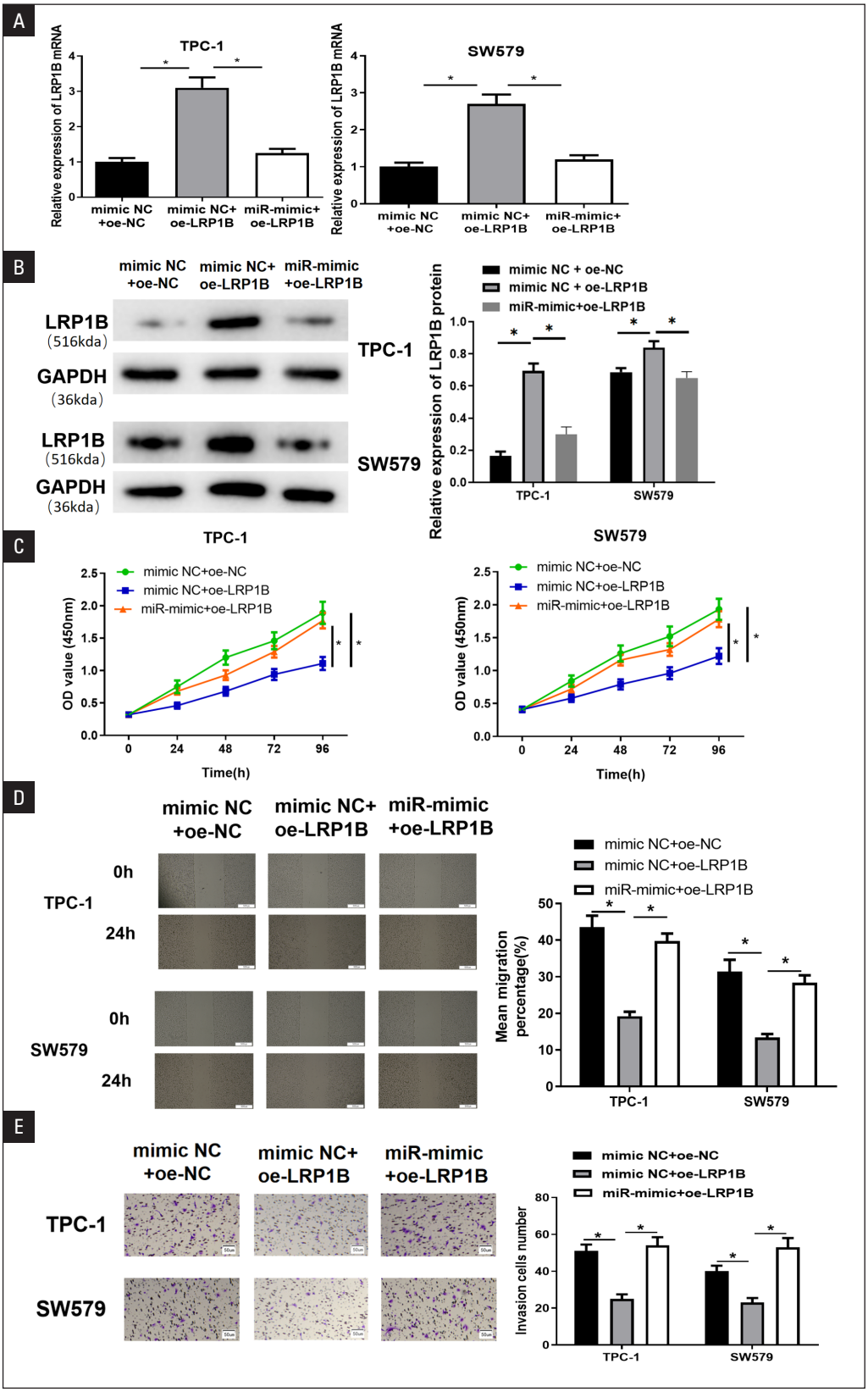

Figure 5. *MicroRNA-196a-5p promotes thyroid cancer (TC) progression by targeting low-density lipoprotein receptor-associated protein 1B (LRP1B).* **AB.** *Expression levels of LRP1B mRNA and protein in TC cells TPC-1 and SW579 transfected with mimic NC+oe-NC, mimic NC+oe-LRP1B, and microRNA-mimic+oe-LRP1B;* **C.** *The proliferative ability of TC cells TPC-1 and SW579 in different transfection groups;* **D.** *The migratory ability of TC cells TPC-1 and SW579 in different transfection groups;* **E.** *The invasive ability of TC cells TPC-1 and SW579 in different transfection groups (100×); *p < 0.05, mimic NC+oe-NC vs. mimic NC+oe-LRP1B, mimic NC+oe-LRP1B vs. microRNA-mimic+oe-LRP1B, the results were expressed as mean ± standard deviation (SD)*

Conflict interest

The authors declare no conflicts of interest.

Funding

Not applicable

Authors' contributions

Y.H, C.Z. conceived and designed the analysis; C.Z. processed and collected the data; Q.C. performed experiments; J.D.: performed the analysis; H.L. collected the data; X.G. drafted the paper; W.C. edited the paper; S.L. revised the paper; C.C. approved the final version.

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